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Proconvulsant-induced seizures in α_4 nicotinic acetylcholine receptor subunit knockout mice

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Abstract

The genetic basis of a number of epilepsy syndromes has been identified but the precise mechanism whereby these mutations produce seizures is unknown. Three mutations of the α_4 subunit of the neuronal nicotinic acetylcholine receptor (nAChR) have been identified in autosomal dominant nocturnal frontal lobe epilepsy. In vitro studies of two mutations suggest an alteration of receptor function resulting in decreased ion channel current flow. We investigated the response of α_4 nAChR subunit knockout mice to the γ-aminobutyric acid (GABA) receptor antagonists; pentylenetetrazole (PTZ) and bicuculline (BIC), the glutamate receptor agonist kainic acid (KA), the glycine receptor antagonist strychnine and the K^+ channel blocker 4-aminopyridine (4-AP). Mutant (Mt) mice had a greater sensitivity to PTZ and BIC, with an increase in major motor seizures and seizure-related deaths. Furthermore, Mt mice were more sensitive to KA and strychnine, but the effects were much smaller compared to those seen with the GABA receptor antagonists. Paradoxically, Mt mice appeared to be relatively protected from 4-AP-induced major motor seizures and death.

The results show that a functional deletion of the α_4 nAChR subunit in vivo is associated with a major increase in sensitivity to GABA receptor blockers.

Keywords: α_4 nicotinic knockout; Seizures; Pentylenetetrazole; Bicuculline; Kainic acid; Strychnine

1. Introduction

Epilepsy is a condition that affects 2–3% of the population at some time in their lives. The epilepsies are broadly grouped into two etiological categories, symptomatic and idiopathic. The seizures that occur in symptomatic epilepsies result from gross disturbances of neuronal physiology, typically associated with progressive death of neurons and brain atrophy. In contrast, genetic factors are believed to be paramount in the idiopathic epilepsies and offer the chance to study epileptogenesis at the mechanistic level as the mutations appear to selectively interfere with seizure threshold, without significantly disturbing other neuronal functions. Significant headway has been made in identifying mutations underlying some rare forms of inherited epilepsy (Steinlein et al., 1995, 1997; Wallace et al., 1998; Hirose et al., 1999; Phillips et al., 2001); the task is now to explain how these mutations produce the epileptic phenotype. Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is a recently recognized syndrome in which brief partial seizures occur during light sleep and often are misdiagnosed as nightmares. Three mutations in the α_4 subunit of the neuronal nicotinic acetylcholine receptor (nAChR) have been described in families with ADNFLE (Steinlein et al., 1995, 1997; Hirose et al., 1999) and, more recently, mutations have been described in the β_2 nAChR subunit (Fusco et al., 2000; Phillips et al., 2001).

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A large number of structurally related nAChRs are expressed in excitable tissues; they are pentameric ligand gated ion channel receptors variously composed of α subunits (α_{2-10}) and β subunits (β_{2-4}) (Le Novere and Changeux, 1995; Elgoyhen et al., 2001). The $\alpha_4\beta_2$ receptor configuration is expressed at high levels and is thought to play an important neuromodulatory role in anxiety (Ross et al., 2000), cognition, neurodegeneration (Brioni et al., 1997; Ryan et al., 2001) and antinociception (Damaj et al., 1998; Marubio et al., 1999). The α_4 transcripts are detected in many brain regions that are potentially relevant to epileptogenesis including thalamus, cerebral cortex and hippocampus (Wada et al., 1989). At the cellular level, the $\alpha_4\beta_2$ nAChR has been shown to facilitate the release of a number of neurotransmitters including dopamine and γ-aminobutyric acid (GABA) (Role and Berg, 1996) and to excite cortical GABAergic interneurons (Porter et al., 1999; Alkondon et al., 2000). Two mutations identified in the α_4 subunit in ADNFLE involve amino acid substitutions [Ser248-Phe (Steinlein et al., 1995) and Ser252Leu (Hirose et al., 1999)] and one involves a leucine insertion [259insLeu (Steinlein et al., 1997)]. All three mutations are located in the second transmembrane domain that lines the ion channel pore of the receptor (Akabas et al., 1994; Steinlein et al., 1995), which suggests that they may have major adverse effects on channel function. For the Ser248Phe and 259insLeu mutations, this is supported by in vitro examination of *Xenopus* oocytes expressing the mutated α_4 nAChR subunits; functional studies of the Ser252Leu mutation are yet to be reported. A number of *Xenopus* oocyte studies have examined the in vitro effects of mutations identified in ADNFLE families (Weiland et al., 1996; Kuryatov et al., 1997; Bertrand et al., 1998; Figl et al., 1998). These studies identify the net effect of α_4 nAChR subunit mutations associated with ADNFLE as reduced current flow through the mutant receptor, faster desensitization of the receptor upon activation by acetylcholine and greatly reduced Ca^{2+} permeability; features that suggest receptor hypofunction. However, a notable feature is use-dependent potentiation that theoretically may be capable of establishing high frequency cholinergic input to presynaptic terminals and thereby trigger seizures.

We used mice with targeted deletion of the α_4 nAChR subunit to address the hypothesis that targeted deletion of the α_4 subunits results in enhanced seizure activity. Homozygous mutant (Mt) mice were generated and characterized in our laboratory (Ross et al., 2000). Seizure activity was assessed in Mt and wild type (Wt) mice in response to two GABA receptor antagonists, pentylenetetrazole (PTZ) (Ramanjaneyulu and Ticku, 1984) and bicuculline (BIC). Kainic acid (KA), strychnine and 4-aminopyridine (4-AP) were chosen to determine whether the nAChR mutants had a nonspecific increased susceptibility to all proconvulsants or displayed a specific $GABA_A$ receptor-mediated alteration in chemoconvulsive threshold.

2. Material and methods

2.1. Animals

All procedures involving the use of live animals conformed to the Australian National Health and Medical Research Council code of practice. Gene knockout and control mice used in the experiments were generated as described previously (Ross et al., 2000). Heterozygous mutant mice that had been backcrossed twice to the C57BL/6 strain were used to generate 10 mating pairs of Mt mice for interbreeding and 10 mating pairs of Wt breeders. The breeding strategy was designed to maximize heterogeneity in the genetic background of mice used in the study. Mating pairs within a genotype were interchanged frequently and randomly so as not to inadvertently select for potential modifier genes. Furthermore, these experiments used a total of 207 Mt and 207 Wt mice and a large number of drugs and drug dosages. In addition, experiments on drugs within the same class (PTZ and BIC) were done at different times. In summary, the experimental design was such that the data is explainable entirely on the basis of biological differences between Mt and Wt mice rather than accidental selection of strain specific modifier genes. Adult mice between the ages of 12 weeks and 15 weeks were used in the seizure and ligand autoradiography experiments. Student's two-sample *t*-test and the Mann–Whitney test were used to test for differences between the number of seizures in each group of mice; in all cases both tests led to similar statistical inferences. All mice used in this study were drug naïve. On experimental days, mice were removed from their home cages and placed individually in clear glass observation cages (36×20×20 cm) for 1 h to habituate prior to administration of each proconvulsant. All proconvulsant experiments were conducted between 0900–1300 h. Types of seizure events were scored immediately after drug treatment and all mice were killed after each experiment to minimize animal suffering. Observers scoring seizure responses were unaware of the genotype of the mice.

2.2. Drug treatment

PTZ, BIC, KA, strychnine and 4-AP were purchased from Sigma (St Louis, MO) and dissolved in 0.9% saline solution. PTZ and KA were administered subcutaneously; BIC, 4-AP and strychnine were given intraperitoneally. Seizures responses were recorded as an average number of individual seizures for any given type and as the 'worst seizure type' where the latter was defined as

the most advanced seizure phase obtained during the observation period for any given animal.

2.3. Pentylenetetrazole-induced seizures

PTZ-induced seizures were studied in females and males in separate experiments. Twenty female Mt mice, (mean body weight 23.9±0.9 g) were compared with 20 female Wt mice (mean body weight 22.7±0.9 g) with respect to their response to a single subcutaneous injection of 80 mg/kg of PTZ. Immediately after the injection, individual mice were observed for 5 s intervals out of every minute cycle for a period of 1 h. PTZ-induced seizures were classified as described by Ferraro et al., (1999). The most commonly observed PTZ-induced seizure began with an arrest of normal exploratory behavior followed by a decrease in motor activity with the animal coming to a complete rest in a crouched or prone position. This hypokinetic seizure type was described as a phase 1 seizure. Phase 2 or partial clonus seizures were characterized by brief twitching movements involving face, head, forelimbs or hind limbs. Phase 3 or generalized clonic seizures occurred when focal twitching was rapidly followed by loss of postural control and repetitive or clonic movements involving all limbs and tail. Jumping and repetitive rearing behavior were also classified as phase 3 activity. A phase 4 or tonic–clonic seizure was characterized by tonic hind limb extension. Phase 4 seizures commonly resulted in death. PTZ-induced seizures were a continuum from phase 1 to phase 4. We also registered time bins in which mice exclusively displayed overtly normal behavior (including rearing, locomotion, grooming, sniffing and climbing) as phase *N* events. Twenty-five male mice of each genotype (Mt mice had a mean body weight 30.4±0.7 g and Wt mice had a mean body weight 30.9±0.6 g) were also tested in an identical PTZ-seizure induction paradigm.

2.4. Bicuculline-induced seizures

We assessed the seizure response to 4 mg/kg of intraperitoneal BIC of 12 male Mt mice (mean body weight 26.5±0.5 g) compared to 12 male Wt control mice (mean body weight 29.2±0.8 g). Mice were observed for 30 min and drug effects scored independently by two observers. Individual mice were observed continuously in two non overlapping 15 s periods by each observer in each 60 s time period and the total score for each mouse/min was determined by adding the two scores. This approach therefore allowed continuous observation of mouse behavior for 50% of the time. The classification of BIC-induced seizures used is as described above for PTZ.

2.5. Kainic acid-induced seizures

KA was administered subcutaneously to mice at a dose of 30 mg/kg to 14 male mice of each genotype. All mice were aged between 12 and 13 weeks and were of similar weights (Wt 27.0±0.9 g, Mt 29.3±0.7 g). Two observers scored drug effects independently over a 90 min period. The observation protocol was as for BIC. As for PTZ seizures, phase *N* events represented normal exploratory mouse behavior. Phases 1 to phase 5 were as described by Yang et al. (1997). In brief, phase 1 involved an arrest of motion, fixed gaze and abnormal forelimb or hind limb posturing. Phase 2 involved myoclonic jerks of head and upper body with associated back arching. Phase 3 involved unilateral clonic activity. Phase 4 involved bilateral synchronous forelimb clonic activity (piano-playing behavior) and phase 5, loss of postural tone and generalized tonic–clonic seizure activity.

2.6. Strychnine-induced seizures

The seizure response to 0.6 mg/kg strychnine of Mt male mice (mean body weight 27.4±0.7 g) was compared to 20 male Wt control mice (mean body weight 28.9±0.8 g). Mice were observed for 30 min and drug effects scored independently by two observers. The observation protocol was as for BIC and KA. The classification of strychnine-induced seizures was as described for PTZ.

2.7. 4-Aminopyridine-induced seizures

The drug 4-AP, a K^+ channel blocker, causes seizures characterized by episodes of running and myoclonic jerks usually terminating in a tonic hind limb extensor convulsion (Yamaguchi and Rogawski, 1992; Cramer et al., 1994). The seizure responses of 20 male Mt mice (mean body weight 27.0±0.7 g) to 10 mg/kg of 4-AP given intraperitoneally were compared to 20 male Wt control mice (mean body weight 29.7±1.1 g). In addition, the seizure response profile of Mt and Wt mice to 12 mg/kg 4-AP was also assessed (Mt, 28.5±0.7 g; Wt, 28.8±0.6 g; all male, $n = 21$ for each genotype). Mice were observed for 90 min and drug effects scored independently by two observers. Individual mice were observed continuously in two non overlapping 10 s periods by each observer in each 60 s time period and the total score for each mouse/min was calculated by adding the two scores. The response profile and scoring methodology was as described for KA, except that phase 3 events after 4-AP involved whole-body tremors.

2.8. Proconvulsant dose responses

Each proconvulsant was examined in male Mt and Wt mice over a range of doses so as to establish dose

response curves. PTZ was given as subcutaneous injections at doses of 65 ($n = 6$), 70 ($n = 6$), 80 ($n = 25$), and 90 ($n = 3$) mg/kg of animal body weight. BIC was given as intraperitoneal injections at doses of 1 ($n = 3$), 2 ($n = 7$), 3 ($n = 7$), and 4 ($n = 12$) mg/kg. KA was given as subcutaneous injections at doses of 30 ($n = 3$), 35 ($n = 7$), 40 ($n = 7$), and 50 ($n = 22$) mg/kg. Strychnine was given as intraperitoneal injections at doses of 0.5 ($n = 4$), 0.6 ($n = 20$), 0.75 ($n = 4$), and 1($n = 5$) mg/kg. 4-AP was given as intraperitoneal injections at doses of 6 ($n = 4$), 8 ($n = 4$), 10 ($n = 4$), and 12 mg/kg ($n = 21$).

2.9. $GABA_A$ receptor binding

The $GABA_A$ receptor antagonist, [^{3}H]SR95531 (NET-946, New England Nuclear, Boston, MA) was used to characterize the distribution of ionotropic GABA receptors in Wt and Mt mouse brains. Frozen slide-mounted 20 μm coronal sections from drug naïve mice were thawed at room temperature before pre-incubation in 50 mM Tris/citrate buffer (pH 7.4) containing 100 mM $MgCl_2$. The sections were cooled in ice-cold buffer for five min before incubation in 6.5 nM [^{3}H]SR95531 in the same buffer for 30 min. The sections were washed three times in ice-cold buffer for 5 s and rinsed in distilled water twice for 10 s before drying. Non-specific binding was determined in the presence of 10 mM GABA (Research Biochemicals International, MA). Autoradiographic detection was carried out by exposing the slide-mounted sections, together with [^{3}H]microscales (RPA 510, Amersham International, UK) to Hyperfilm (RPN12, Amersham) for 12 days. The films were developed using Kodak D-19 Photo Developer. Binding densities were measured using a microcomputer imaging device (MCID) with software (Imaging Research Inc. Brock University, St. Catherine's, Ont., Canada). For all studies, a minimum of three coronal sections from each animal was used for calculation of individual means. Standardization was achieved by comparing binding densities with standards exposed with each film. All values are expressed as mean±SEM (fmol/mg). Student *t*-tests were used for statistical analysis of autoradiographic regional quantitative binding densities.

3. Results

Mt mice demonstrated hyperactive home cage behavior from weaning time but did not exhibit spontaneous clinical seizure activity. Mt mice did not die unexpectedly indicating that major nocturnal motor seizures were unlikely. Vehicle treated Mt and Wt mice did not exhibit clinical seizure activity.

3.1. Pentylenetetrazole-induced seizures

Following PTZ administration, female Mt mice showed less normal behavior ($P = 0.0001$), a reduction in the number of phase 1 ($P = 0.02$) events, a comparable number of phase 2 and phase 3 events and an increase in phase 4 ($P = 0.01$) events (Fig. 1A). There was also a significant increase in the death rate of female Mt mice, with 65% of mutants dying from seizures compared to 15% in Wt mice (Fishers exact test $P = 0.002$) (Table 1A). This was comparable to a death rates of 79% in Mt mice compared to 13% in Wt mice obtained in an independent experiment examining an equal number of male and female mice of each genotype (Mt; $n = 14$, Wt; $n = 15$) (Fishers exact test $P = 0.002$). Furthermore, death did not inevitably follow phase 4 events in either genotype (see Table 1A). Male mice tested using an identical paradigm showed a similar reduction in normal behavior and phase 1 events and showed a trend to an absolute increase in the average number of phase 4 events ($P = 0.06$) (Fig. 1B). There was, however, a significant increase in seizure associated deaths in male Mt mice ($P = 0.021$) (Table 1B). The relative reduction in the absolute number of phase 4 events in male mice compared to female mice may relate in part to the earlier time of onset of death in Mt males in comparison to female Mt mice (average time of onset of death in Mt females was 25±2.5 compared to 20±1 min in Mt males). The data as presented does not indicate a gender difference with respect to the response of Mt mice to PTZ as measured by seizure associated death as the endpoint.

3.2. Bicuculline-induced seizures

Mt mice showed less normal behavior ($P = 0.022$), a reduction in phase 1 ($P = 0.001$) and phase 2 ($P = 0.024$) events, a comparable number of phase 3 and an increased number of phase 4 events ($P = 0.032$) (Fig. 1C). Unlike PTZ-induced seizures, tonic–clonic events following BIC injection were associated with death resulting in a mortality of 92% in Mt mice compared to 33% in Wt controls (Fishers exact test $P = 0.009$) (Table 1C).

3.3. Kainic acid-induced seizures

KA administration resulted in a six-fold reduction in normal exploratory behavior in Mt as compared to Wt mice (average number of observation±SEM/mouse/90 min: Wt; 64.5±10, compared to 11.0±4.0 in Mt mice, $P = 0.0003$) (Fig. 1D). In addition, Mt mice had a greater number of phase 1 ($P = 0.002$) and phase 2 events ($P = 0.001$). There was no significant difference between genotypes with respect to the number of phase

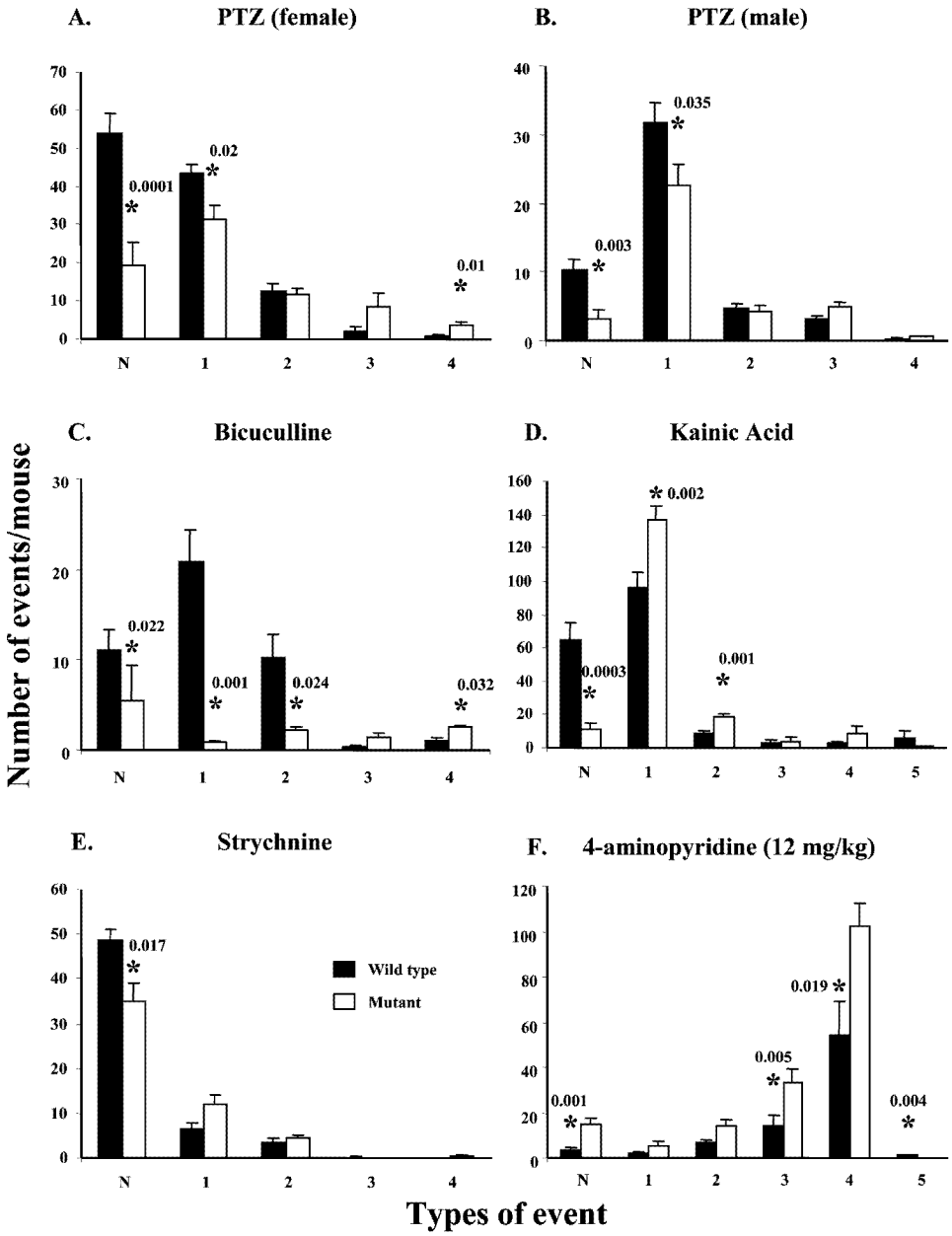

Fig. 1. The number of events/mouse according to type in Wt and Mt mice during the observation period for each drug tested. (A) PTZ females, (B) PTZ males, (C) BIC, (D) KA, (E) strychnine and (F) 4-AP (12 mg/kg). Filled bars represent Wt and empty bars Mt. *Significant P values when compared to Wt mice.

3, 4, or 5 events, although seven of the Mt mice (50%) had a phase 5 seizure compared to only 2 (14%) in Wt mice (Table 1D).

3.4. Strychnine-induced seizures

Mt mice showed less normal behavior ($P = 0.017$) but no change in the number of other events (Fig. 1E). Three of the drug-treated Mt mice died while there were no deaths in the Wt control group (Fisher exact test $P = 0.231$) (Table 1E).

3.5. 4-Aminopyridine-induced seizures

Both genotypes showed a complete spectrum of seizure types (see Fig. 1F for response to 12 mg/kg) with all mice progressing to major motor seizures (include phase 4 and phase 5)(Table 2). In contrast to PTZ, BIC, KA and strychnine induced seizure activity, we found that 4-AP tended to produce infrequent hypokinetic and minor motor seizures and rapid progression to major motor seizures and death especially in Wt mice. Wt mice showed a paradoxical decrease in phase 3 and phase 4

Table 1
Seizure types

	Phase *N*	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Deaths
(A) Worst PTZ-induced seizure type (females)							
Wt (n = 4)	0 (0%)	1 (5%)	7 (35%)	8 (40%)	4 (20%)		3 (15%)
Mt (n = 4)	0 (0%)	0 (0%)	0 (0%)	6 (30%)	14 (70%)		13 (65%)***
(B) Worst PTZ-induced seizure type (males)							
Wt (n = 25)	0 (0%)	0 (0%)	3 (12%)	15 (60%)	7 (28%)		6 (24%)
Mt (n = 25)	0 (0%)	0 (0%)	0 (0%)	9 (36%)	16 (64%)		15 (60%)*
(C) Worst BIC-induced seizure type							
Wt (n = 12)	0 (0%)	0 (0%)	7 (58.3%)	1 (8.3%)	4 (33.3%)		4 (33.3%)
Mt (n = 12)	1 (8%)	0 (0%)	0 (0%)	0 (0%)	11 (92%)		11(92%)**
(D) Worst KA-induced seizure type							
Wt (n = 14)	0 (0%)	0 (0%)	5 (35.7%)	4 (28.6%)	3 (21.4%)	2 (14.3%)	0 (0%)
Mt (n = 14)	0 (0%)	0 (0%)	1 (7.1%)	3 (21.4%)	3 (21.4%)	7 (50%)	0 (0%)
(E) Worst strychnine-induced seizure type							
Wt (n = 4)	0 (0%)	4 (20%)	15 (75%)	1 (5%)	0 (0%)		0 (0%)
Mt (n = 4)	1 (5%)	1 (5%)	15 (75%)	0 (0%)	3 (15%)		3 (15%)

Distribution of mice according to the worst observed seizure event, expressed as the number of mice (and % of each genotype) in each seizure category. Refer to main text for the description of the types of seizure events. The numbers of animals dying after drug treatments were included in the last column, showing that not all animals died after major motor seizures.

* P = 0.001.

** P = 0.009.

*** P = 0.002, Fisher Exact test.

Table 2
Worst 4-aminopyridine-induced seizure type

	Phase *N*	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Deaths
(A) 10 mg/kg							
Wt(n = 4)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	13/20 (65%)	7/20 (35%)	5/20 (25%)*
Mt(n = 4)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	20/20 (100%)	0/20 (0%)	0/20 (0%)
(B) 12 mg/kg							
Wt(n = 21)	0/21 (0%)	0/21 (0%)	0/21 (0%)	0/21 (0%)	7/21 (33.3%)	14/21 (66.7%)	14/21 (66.7%)**
Mt(n = 21)	0/21 (0%)	0/21 (0%)	0/21 (0%)	0/21 (0%)	19/21(90.5%)	2/21 (9.5%)	2/21 (9.5%)

Distribution of mice according to the worst observed seizure event, expressed as the number of mice (and % of each genotype) in each seizure category. Refer to main text for the description of the types of seizure events. The number of animals dying after drug treatments is included in the last column.

* P = 0.043.

** P = 0.0003, Fisher Exact test.

events (Fig. 1F) presumably due to drop out of mice from the data set as a consequence of early death (average time of death was 12 min in Wt and 40 min in Mt mice). Twenty five percent (5 out of the 20) of Wt mice treated with 10 mg/kg of 4-AP died while there was no death in the Mt group (Fisher exact test P = 0.043). For mice treated with 12 mg/kg of 4-AP, 14 out of 21 (66.7%) Wt mice progressed to phase 5 seizures and died while only 2 Mt out of 21 (9.5%) had phase 5 seizures and also died (Fisher exact test P = 0.0003) (Table 2).

3.6. Proconvulsant dose responses

There is a clear difference between Wt and Mt mice when final death rate is used as the experimental end point (Fig. 3). The death rate was higher in Mt for all doses of PTZ tested (65–90 mg/kg) and for 3 and 4 mg/kg for BIC. In contrast, 10 and 12 mg/kg of 4-AP gave substantially higher death rates in Wt mice. There was no consistent pattern in relative death rates for KA and strychnine.

3.7. $GABA_A$ receptor binding

The brain regions analyzed were: cortex at level of striatum (Cx), caudate/putamen (CPu), nucleus accumbens (NAc), olfactory tubercle (T), lateral globus pallidus (LPG), interpeduncular nucleus (IPn), lateral septum (LS), periaqueductal grey (PAG), pontine nucleus (Pn), anterior paraventricular thalamus (Pva), substantia nigra pars reticulata (SNr) and superior colliculus (SC).

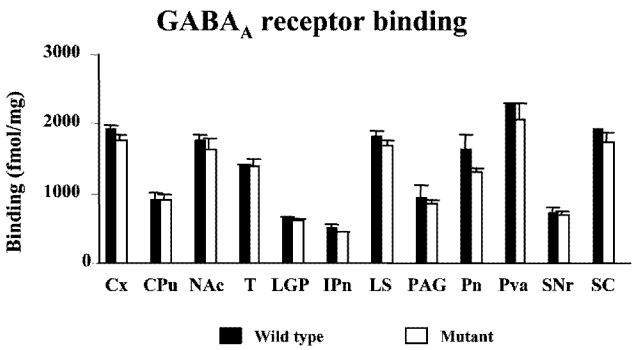

Fig. 2. Quantitative $GABA_A$ receptor binding in WT and Mt brains. Brain regions analyzed were: cortex (Cx), caudate/putamen (CPu), nucleus accumbens (NAc), olfactory tubercle (T), lateral globus pallidus (LGP), interpeduncular nucleus (IPn), lateral septum (LS), periaqueductal grey (PAG), pontine nucleus (Pn), anterior paraventricular thalamus (Pva), substantia nigra pars reticulata (SNr) and superior colliculus (SC). There were no significant differences in quantitative ligand binding between Wt and Mt brains for any of the brain regions examined (Student's *t*-tests).

There were no significant differences between Wt and Mt mice in the density of $GABA_A$ binding sites in any regions assessed (Fig. 2).

4. Discussion

We investigated the seizure responses of α_4 nAChR subunit knockout mice to PTZ, BIC, KA and strychnine. Mice with targeted deletion of the α_4 nAChR subunit showed a substantial increase in severity of GABA blocker-induced seizures compared to controls. Both PTZ and BIC produced a reduction in mild hypokinetic seizure events and an increase in the incidence of major motor seizures and seizure-related deaths. The effects of the proconvulsants on Mt mice are unlikely to be due to compensatory changes in non-α_4 nAChR subunits as we observed no change in α_3, α_6, α_7, β_2, β_3, and β_4 Mt brain mRNA levels (Ross et al., 2000).

The observation that many proconvulsant agents reduce CNS GABA activity and a number of anti-convulsant drugs enhance the activity of GABAergic neurotransmission (Olsen, 1981; White, 1999) provides support for the idea that down-regulated CNS GABAergic neurotransmission is important in seizure generation and propagation. In addition, mutations have been described in the γ_2 subunit of the GABA receptor of families with an idiopathic epilepsy phenotype (Baulac et al., 2001; Wallace et al., 2001). Recent evidence suggests that the principal function of the nAChRs may be to modulate presynaptic release of other neurotransmitters including GABA, dopamine, acetylcholine, noradrenaline and glutamate (Role and Berg, 1996). There are a number of studies describing nicotine-induced release of the inhibitory neurotransmitter GABA either from isolated synaptosomes or in slice preparations (Lena et al., 1993; Kayadjanian et al., 1994). It seems likely that $\alpha_4\beta_2$ receptor dysfunction, whether due to the presence of channel pore mutations or to targeted deletion of the α_4 nAChR subunit, leads to disruption of presynaptic transmitter release mechanisms. As both PTZ and BIC produce seizures by blockade of brain GABA receptors, we postulate that the marked sensitivity of Mt mice to PTZ may reflect reduced steady state levels of GABAergic inhibitory tone. Neurotransmitter receptor and transporter knockout mice are known to undergo a number of secondary compensatory changes in associated neurotransmitter systems (Drago et al., 1998). Normal GABA receptor binding in drug naïve Mt mice suggests that enhanced sensitivity to GABA receptor blockade is not related simply to compensatory down-regulation of GABA receptor expression.

Unlike the marked increased sensitivity to PTZ and

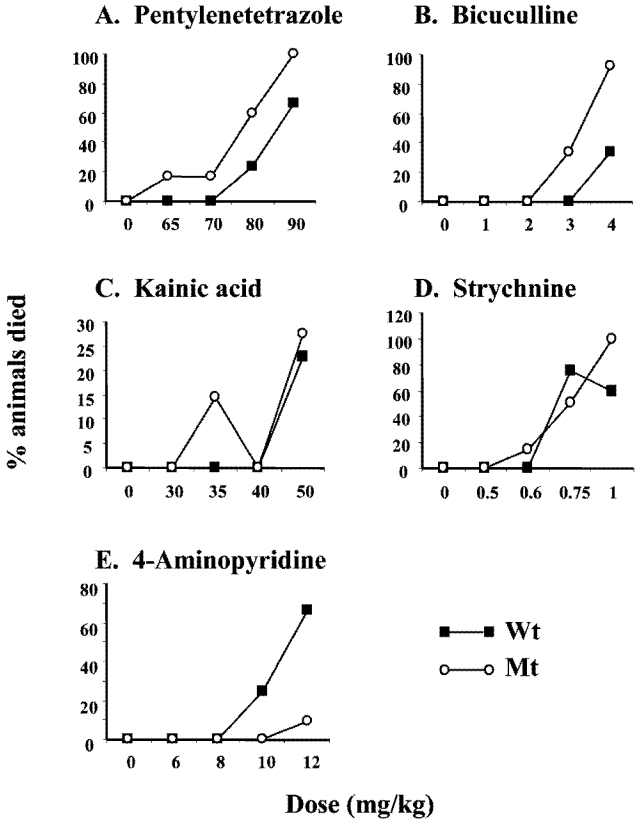

Fig. 3. Dose–response curves for proconvulsant associated death rate. (A) PTZ was given as subcutaneous injections at doses of 65 (n = 6), 70 (n = 6), 80 (n = 25), and 90 (n = 3) mg/kg of animal body weight. (B) BIC was given as intraperitoneal injections at doses of 1 (n = 3), 2 (n = 7), 3 (n = 7), and 4 (n = 12) mg/kg. (C) KA was given as subcutaneous injections at doses of 30 (n = 3), 35 (n = 7), 40 (n = 7), and 50 (n = 22) mg/kg. (D) Strychnine was given as intraperitoneal injections at doses of 0.5 (n = 4), 0.6 (n = 20), 0.75 (n = 4), and 1(n = 5) mg/kg. (E) 4-AP was given as intraperitoneal injections at doses of 6 (n = 4), 8 (n = 4), 10 (n = 20), and 12 mg/kg (n = 21). None of the saline-treated (10 ml/kg, intraperitoneally) mice (n = 5 for each genotype) died.

BIC, the response to the dose of KA used in this study was less marked, although a clear difference between genotypes was apparent. Compared to Wt mice, Mt mice showed a significant reduction in normal behavioral counts and an increase in the number of hypokinetic and minor motor seizures. The response to KA confirms that Mt mice show an increased sensitivity to proconvulsants, other than those acting primarily through the GABAergic system. Nicotinic receptors have been shown to modulate excitatory amino acid (EAA) neurotransmission at a number of sites in the CNS. Presynaptic nicotinic receptors are present on thalamocortical projections where they are thought to regulate glutamate release from presynaptic terminals (Gioanni et al., 1999) as well as augment [^{3}H]D-aspartate efflux in primary cultures of cortical neurons (Beani et al., 2000). The response of Mt mice to KA, however, is the opposite from what would be predicted if the principal role of α_4nAChR subunit containing nicotinic receptors were facilitation of neocortical EAA release. There are a number of possible explanations of the observed sensitivity of Mt mice to KA. First, post-synaptic receptors containing the α_4 nAChR subunit have been identified in a subpopulation of cortical GABAergic interneurons (Porter et al., 1999) and altered cortical excitability resulting from loss of the receptor in this cell population may underlie the KA response. Second, there may be developmentally determined compensatory changes in glutamate receptor kinetics or downstream signaling mechanisms. The third and most plausible explanation, particularly given our observations with PTZ and BIC, is that there is failure of the Mt brain to up-regulate GABA release following glutamatergic challenge. In other words, what may be important is the balance between perturbations to GABAergic and EAA neurotransmission, a concept that has been elaborated by Ben-Ari and Cossart (2000).

The seizure response to the dose of strychnine used in this study was the same in both genotypes although there was a highly significant difference in the reduction of normal exploratory behaviour in Mt mice. There was also a trend towards an increase in death rate in Mt mice. As we postulated for KA-induced seizures, the response of Mt mice to strychnine may also be explainable by failure to upregulate GABAergic tone.

The death rate and seizure profile seen in Mt mice following 4-AP was significantly less than seen in Wt mice. This result suggests that Mt mice are paradoxically protected from 4-AP induced seizures. The proconvulsant 4-AP stimulates the release of glutamate from synaptic terminals and the increased extracellular glutamate precipitates epileptiform discharges by over activation of glutamate receptors (Medina-Ceja et al., 2000; Pena and Tapia, 2000). We postulate that Mt mice may have compensated for a GABAergic defect by down regulating presynaptic glutamatergic processes.

In conclusion, the observation of enhanced sensitivity to a variety of proconvulsants in α_4 nAChR subunit knockout mice is consistent with the idea that this induced mutation is associated with a generalized activation of neuronal activity. The heightened anxiety-like behavior and elevation of unconditioned motor activity observed in Mt mice (Ross et al., 2000) is also consistent with this hypothesis. In vitro studies of the α_4 nAChR subunit mutations identified in human ADNFLE have previously suggested that nicotinic receptor dysfunction may be responsible for the epilepsy phenotype. These studies have suggested that either reduced acetylcholine receptor function on presynaptic terminals results, in some way, in impaired inhibitory processes or that use-dependent potentiation is capable of establishing high frequency cholinergic input to presynaptic terminals thereby trigger seizures by augmented release of proconvulsant neurotransmitters. The current study shows that the null mutation is associated with abnormal excitability and a heightened proconvulsant-induced seizure response particularly to drugs acting through the GABAergic system and a paradoxical decreased response to 4-AP, an agent that is thought to cause seizures by the release of glutamate. The conclusion is that in our model and possibly in ADNFLE itself that the likely in vivo consequence of a hypo-functioning α_4 nAChR allele is an increase in neuronal excitability.

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